The Use of Reverse Line-Blot Hybridization for the Detection of Trichostrongylid Nematodes in Small Ruminants

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I. Abstract

Helminth infections are among the top causes of economic loss in the upkeep of small ruminants, and with anthelmintic resistance on the rise, it is vital that rapid and versatile diagnostic tests be developed to better inform farmers and veterinarians and to assist in their treatment decisions. In this study, reverse line-blot hybridization is explored for the genus-specific detection of three major abomasal parasites of sheep and goats: *Haemonchus, Trichostrongylus,* and *Teladorsagia.* Using previously developed genus-specific primers complementary to internal transcribed spacer 2 (ITS-2), this assay allows for the specific detection of *H. contortus* and the nonspecific detection of the remaining two abomasal parasites using a cross-hybridizing probe. Although additional research is needed to improve assay specificity, the technique explored here provides a potential high-throughput alternative for the detection of *H. contortus*, and shows promise for future applications in the detection of anthelmintic resistance-conferring single nucleotide polymorphisms (SNPs).

II. Introduction

Helminth infection is considered by many to be the single most economically important infectious disease of small ruminants [1]. Global estimates are lacking, but individual countries have estimated their own losses to be anywhere from US$ 42M per year in Uruguay to US$ 222M per year in Australia [1,2] In the developed world, a considerable portion of these costs can be attributed to treatment expenses, however the bulk of these financial losses, particularly in the developing world, is the result of lost productive potential due to subclinical disease [2-4]. With high prevalence levels reported across the globe in the United States [5], Nigeria [6], India [7], and Serbia [8], to name a few, helminth infections are of clear economic and global importance in the modern world.

Although many different species of nematodes are known to parasitize sheep and goats, the majority of these infections are caused by the members of three genera: *Haemonchus, Trichostrongylus*, and *Teladorsagia* [9]. Each varies in its pathogenesis, but all have very similar life cycles. These parasites begin their journey passed in the feces of an infected animal as an egg containing the morula developmental stage. After 1-2 days of development, a first-stage larva (L1) hatches from the egg and continues to develop in the soil as a free-living microbivorous larva. The developing strongylid then undergoes two more molts before finally becoming a non-feeding, infectious, third-stage larva (L3). At this point, the larva may be ingested by its new host, where it will travel to the abomasum to develop into adulthood and to repeat the cycle [9].

H. contortus is the primary source of loss in populations of small ruminants [5]. Disease stems from the blood meals of the fourth-stage larvae (L4) and adult worms, and the induced signs include hemorrhagic anemia, edema, decreased wool and muscle mass, and in extreme cases, death [5,9]. The lack of gastrointestinal signs, however, can make infection difficult to

detect [5,9]. Alternatively, *Trichostrongylus* and *Teladorsagia* are much more closely associated with gastrointestinal disease. Trichostrongylosis is caused by a number of *Trichostrongylus* species, including *T. axei, T. colubriformis, T. rugatus,* and *T. vitrines* [10,11]. Unlike the other two genera, and with the exception of *T. axei*, *Trichostrongylus* species reside in the small intestine, with heavy infections leading to severe diarrhea, hypoproteinemia, weight loss, and/or death. *T. axei*, which resides in the abomasum, is less commonly associated with clinical disease [5,9]. Finally, *Teladorsagia* (*Ostertagia*) larvae develop in the gastric glands, creating nodules in the abomasum mucosa and causing damage to parietal cells. Although less pathogenic than the other two genera [12], moderate infections commonly lead to diarrhea and poor weight gain. Severe infections may result in anemia, hypoproteinemia, or death [5,9]. In all cases, the animals at most risk of developing helminth infections are the young, the immune-compromised, and those living in environments highly contaminated with infectious L3s [9].

There are currently three major classes of anthelmintics used for the treatment of helminth infections: the benzimidazoles, the imidothiazoles, and the avermectin-milbemycins (macrocyclic lactones). Introduced in the early 1960s, benzimidazoles were quickly adopted for their low cost, ease of administration, broad specificity, and low mammalian toxicity [13,14]. However, heavy use of these drugs placed tremendous selective pressure on anthelmintic resistant organisms, and resistance phenotypes rapidly accumulated in populations of *H. contortus*, *Teladorsagia circumcincta,* and *Trichostrongylus colubriformis* [14]. Now, following the introduction of imidothiazoles in the 1970s and macrocyclic lactones in the 1980s, multiple drug resistance to all major anthelmintic classes has been reported [14]. Novel anthelmintic classes have had limited success. In 2009, Novartis New Zealand released Zolvix®, a broad-spectrum oral anthelmintic containing the amino-acetonitrile derivative monepantel [15,16]. By

2013, however, resistance had been detected in populations of *T. circumcincta* and *T. colubriformis* [16]. Other new treatments, such as the combined use of derquantel and abamectin, are beginning to show decreased efficacy as well [17]. As such, the greatest challenge in the control of helminth infections appears to be when, not if, resistance to novel anthelmintics will develop.

To better manage these increasingly resistant populations, the extent of resistance will need to be determined. This can be assessed by a number of *in vivo* and *in vitro* assays, including fecal egg count reduction tests (FECRT), egg hatch tests, larval development assays, and migration inhibition assays [18, 19]. Of these, FECRT is the most common test of anthelmintic resistance, and involves the microscopic calculation of helminth eggs per gram of feces (epg) before and after host treatment [18]. Although endorsed by the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) [20], FECRT is severely limited in its ability to differentiate between resistant genera. This is largely because of extensive morphological similarities between strongyle eggs, making genus-specific identification impossible with the naked eye. Larval development assays, however, can make up for this lack of specificity. In this assay, eggs are cultured in varying concentrations of anthelmintic, and allowed to develop into third-stage larvae before counting [18]. Although L3s can be differentiated by genus, this technique has the significant drawback of being incredibly time-consuming, with each culture taking up to two weeks to mature [21]. Furthermore, developmental differences between species in culture may generate bias when assigning species identities to mixed egg samples [22]. More rapid and specific diagnostic tools are therefore urgently needed.

Promising higher-throughput analyses and more rapid diagnoses, molecular techniques like real-time and multiplex PCR have become increasingly popular in recent years [9,23]. DNA blotting assays have also been explored for this purpose, and reverse line-blot hybridization (RLBH) has been highlighted for its versatility, low-cost, and high-throughput capabilities [24]. Similar blotting assays for the detection of trichostrongylids have been explored in the past [21]; however, RLBH's dramatically shorter hybridization time and ability to exploit non-radiometric detection methods make the technique attractive for both genus-specific diagnosis, and the detection of specific resistance genotypes. The technique uses oligonucleotide probes fixed to a nylon membrane to selectively hybridize amplified and tagged sample DNA. Properly hybridized samples can then be visualized with radioactive, chemiluminescent, or colorimetric detection methods (Figure 1) [25]. Work has already been done to identify primers for genus-specific amplification of the second internal transcribed spacer (ITS-2), and I hypothesize that these primers may be sufficient for genus-specific detection of *H. contortus, T. axei,* and *T. circumcincta* in a reverse line-blotting assay [26]. In this study, I assess the utility of these primers for that purpose, in the hopes of developing an easily interpretable assay for rapid diagnosis and characterization of helminth infections in small ruminants.

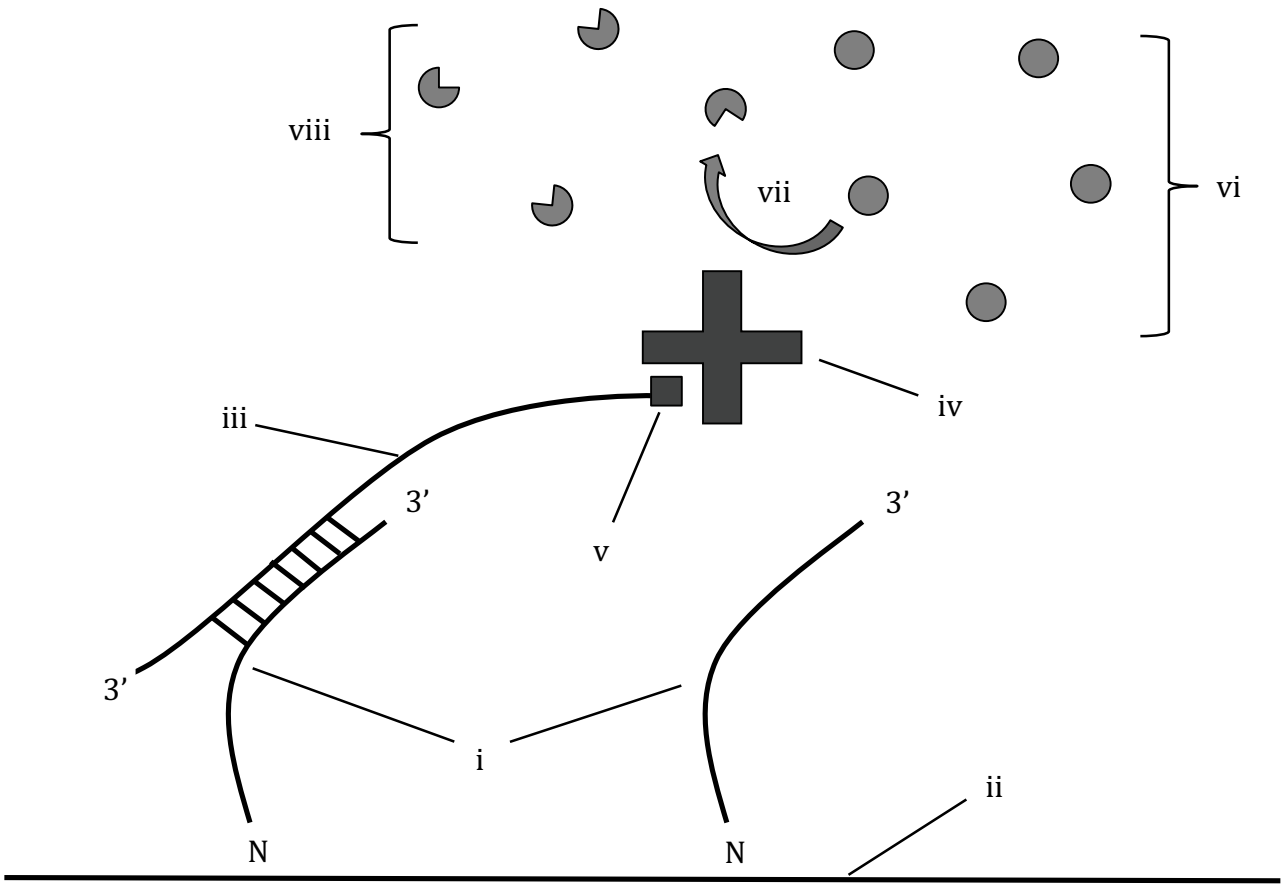

Figure 1. Schematic of reverse line-blot hybridization. Oligonucleotide probes (i) are fixed to a negatively-charged nylon membrane (ii) via a 5' amino group. A 5' biotinylated PCR product (iii) is then hybridized to the oligonucleotide probe and treated with a streptavidin-peroxidase (POD) enzyme conjugate (iv). The streptavidin-POD binds to biotin (v) and cleaves tetramethylbenzidine (TMB) (vi, vii), producing a colored precipitate (viii) in the presence of properly hybridized samples.

III. Materials and Methods

1. Sample Acquisition

Adult worms of the genera *Haemonchus, Trichostrongylus,* and *Teladorsagia* were obtained from the abomasa of slaughtered sheep and lambs. Each abomasum was washed with tap water into a bucket and the contents were sequentially passed through 2 mm, 850 μm, and 425 μm sieves (US standard sieve 10, 20, and 40). Debris accumulated on each sieve was backwashed with tap water into separate buckets, and contents were transferred in parts to a glass pie dish for selection of individual worms. Adults were morphologically identified and stored at +8℃ in RNAlater.

To obtain *H. contortus* eggs, 19 fecal samples were obtained rectally from ewes with natural helminth infections. Total strongyle worm burdens were quantified microscopically using the McMaster counting technique with $MgSO_4$ flotation solution (1.2 spg) [27]. In samples with an FEC greater than 50 epg, the percentage of *H. contortus* was then determined by staining with peanut agglutinin (PNA) lectin-fluorescein isothiocyanate (FITC) (Sigma-Aldrich), as follows. To concentrate the eggs, 1 gram of feces from each ewe was mixed with a small volume of water and filtered through a piece of cheesecloth. The filtrate was then added to a 16 x 100 mm glass culture tube, filled nearly to the top with water, and centrifuged for 1 minute at 800 x g. Next, the supernatant was decanted and the pellet was re-suspended in sugar flotation solution (1.33 spg), filling the tube until a convex meniscus formed over the rim. An 18 x 18 mm glass coverslip was placed on top, and the tubes were centrifuged at 800 x g for 8 minutes. After centrifugation, the coverslip was rinsed with PBS (pH 7.2) into a microfuge tube to a final volume of 1.5 mL. To prepare for lectin staining, the microfuge tubes were centrifuged at 280 x g for 5 minutes, and the supernatant was aspirated down to a volume of 250 μL. The remaining pellet was subsequently

vortexed and re-suspended in 750 μL diluted PNA-FITC, maintaining a concentration of 5μg/mL sample volume. The re-suspended sample was then incubated for 1 hour, rotating in the dark at room temperature. After incubation, samples were centrifuged for another 5 minutes at 280 x g and the supernatant was aspirated, leaving a volume of 250 μL. To wash away any unbound PNA-FITC, the pellet was then vortexed and brought to a final volume of 1.5 mL with PBS. These wash and centrifugation steps were repeated two more times, omitting the addition of PBS after the final spin in preparation for microscopy. The percentage of *H. contortus* was determined by scoring the first 100 eggs in each sample for the presence or absence of fluorescence using an Olympus BX41 fluorescence microscope at an excitation wavelength of 450-490 nm and a 525 nm emission filter. Five representative samples were selected for further processing based on their percent *H. contortus* content. Eggs from these select samples were collected as before and diluted to a concentration of 100 eggs/200 μL with DI water.

DNA from the individual worms and the 100 egg samples was extracted according to manufacturer protocols (QIAGEN DNeasy Blood & Tissue Kit) and confirmed by amplification with the NC1-NC2 primer set in a 50 μL reaction volume (Table 1) [29]. Each reaction contained 1.5mM PCR buffer (15 mM $MgCl_2$), 250 μM dNTP, 1 μM of each primer, 1 mM $MgCl_2$, 1U Taq Polymerase, and 3μL sample DNA. Samples dwelled for 5 minutes at 94^{o}C, followed by 35 cycles of 1 minute at 94^{o}C, 1 minute at 54^{o}C, and 1 minute at 72^{o}C. The reaction ended with a final dwell at 72^{o}C for 5 minutes (Thermolyne, Amplitron II). PCR amplicons were separated on a 2% (w/v) agarose gel in TBE buffer (0.05M Trizma Base, 0.05M Borric Acid, 1 mM EDTA) for 30 minutes. The species identity of the individual worms was determined both by Restriction Fragment Length Polymorphism (RFLP) analysis and by sequencing at the Cornell Institute of Biotechnology. Samples were cleaved with *Dra1* and *Hinf1*, each for 2 hours

Table 1. Primers used to amplify DNA prior to hybridization. All forward primers are biotinylated at the 5' end for colorimetric detection.

Region Amplified	Primer	Sequence (5'-3')*	T_{m1} † (°C)	T_{m2} ‡ (°C)	Product Size	Reference
β-tubulin isotype 1	(F): S5	B-GCCTGGAACGATGGACTCCGT	62	68	814 bp	32
	(R): AS5	GGCTAACTTGCGAAGATCAGCAT	58	68		
ITS-2	(F): NC1	B-ACGTCTGGTTCAGGGTTGTT	55	60	320-340 bp	28
	(R): NC2	TTAGTTTCTTTTCCTCCGCT	51	56		

* B = biotin; † Calculated with the McConaughy equation; ‡ Calculated with the Wallace equation [29].

Table 2. Probes used in reverse line-blot hybridization to detect products amplified by the primers listed in Table 1. The control probe is the reverse complement of S5 (Table 1), while nITS2HC, nITS2T, and nITS2Te are the reverse complement of forward primers used previously for genus-specific PCR amplification. All probes received a 5' amino group for adherence to the blotting membrane.

Primers	Species	Probes	Sequence (5'-3')	%GC	T_m § (°C)	Reference
S5, AS5	*H. contortus*	Control	NH_2-ACGGAGTCCATCGTTCCAGGC	62	70	N/a
NC1, NC2	*H. contortus*	nITS2HC	NH_2-CAATGTTGAAATTAGCCCTC	40	59	26
	T. axei	nITS2T	NH_2-AGAGTTAGCCACACTGTAGAA	43	62	
	Tel. spp.	nITS2Te	NH_2-AGTAATAAATACCATTCGAC	30	55	

§ T_m= 81.5 + 16.6(log M) + 0.41(%GC) - 0.63(% form) - 600/L; *M* = concentration of monovalent cations, *form* = the amount of formamide in the solution, and *L* is the length of the hybrid [30].

at 37°C, and fragments were separated on a 2% (w/v) agarose gel in TBE buffer for comparison to patterns published by Gasser et al. (1994). Sequencing data was edited and aligned using MEGA7 and MegAlign software and compared to the NCBI BLAST database.

To determine if the NC1-NC2 primer pair varied in its ability to amplify DNA of different genera, four adults (2♂, 2♀) from each genus were amplified three separate times. PCR product concentrations (μg/ml) were determined spectrophotometrically at 260 nm (BioRad SmartSpec 3000). For each worm, the three concentration readings were averaged together, and the mean concentration for each genus was compared using one-way analysis of variance (ANOVA). Select fecal samples were amplified only once prior to spectrophotometric analysis. The average adult-derived and egg-derived product concentrations were compared using a two-sample t-test. All statistics were carried out in Minitab 17.3.1 (State College, PA).

PCR product concentrations were converted to pmol using expected PCR product lengths (NCBI Primer BLAST) and the equation:

$$\mu g\ DNA \times \frac{pmol}{660pg} \times \frac{10^6 pg}{1\mu g} \times \frac{1}{N} = pmol\ DNA$$

where N is the number of nucleotides in the amplicon and $\frac{660pg}{pmol}$ is the average molecular weight of a single base pair [31]. If more than one band was produced in gel electrophoresis, the smallest fragment length was used in calculations to return the maximum pmol DNA recovered.

2. Primer and Probe Design

The β-tubulin isotype 1 gene of *H. contortus* was arbitrarily selected for proof of concept and used as a positive control. Individual *H. contortus* adults were amplified with the biotinylated S5-AS5 primer pair in a 50 μL reaction volume (Table 1) [32]. The reaction contained 1.5mM PCR buffer (15 mM $MgCl_2$), 200μ M dNTP, 0.5 μM of each probe, 1.5 mM

$MgCl_2$, 1U Taq Polymerase, and 1 μL sample DNA. Samples dwelled for 5 minutes at 94^{o}C, followed by 35 cycles of 30 seconds at 94^{o}C, 30 seconds at 54^{o}C, 30 seconds at 72^{o}C, and ending with an 8 minute dwell at 72^{o}C. The corresponding probe was designed complementary to the S5 primer and contained a 5' amino group for attachment to the blotting membrane (Table 2). For genus-specific identification, the biotinylated NC1-NC2 primer pair was again used for amplification of DNA from *H. contortus, T. axei,* or *T. circumcincta* worms and eggs (Table 1) [29]. Probes were adapted from the forward primers previously used in genus-specific amplification of the ITS-2 region (Table 2) [26]. Each ITS-2 probe was modified to be the reverse complement of the primer so as to hybridize with the biotinylated NC1-primed DNA strand. Again, all probes contained a 5' amino group for fixation to the membrane.

3. Membrane Preparation and Hybridization

The line-blotting protocol was adapted from those previously published by Kong & Gilbert (2006), Traversa et al. (2007), and Kamst-van Agterveld & Zwart (2002). A 7 x 8.5 cm Biodyne C membranes (0.45 μm, Pall Life Sciences) and three pieces of 8.5 x 13.5 cm Immobilon Blotting Filter Paper (Millipore) were cut to fit the support template of the Bio-Dot SF Microfiltration Apparatus (BioRad, Figure 2). The membrane was then activated in a sealed plastic bag with 10 mL 16% (w/v) EDAC for 10 minutes, and rinsed with DI water. The filter paper was also wet with DI water and placed on the gasket support plate. The rinsed membrane was then laid on top and the support template was tightly screwed into place. Excess liquid was removed by vacuum aspiration through the membrane using flow valve setting 1 to supply maximum vacuum pressure.

To prepare the probes, 0.5 μL of probe at the desired concentration was mixed with 1 mL 500 μM $NaHCO_3$ (pH 8.4). 200 μL of this solution were added to each well according to

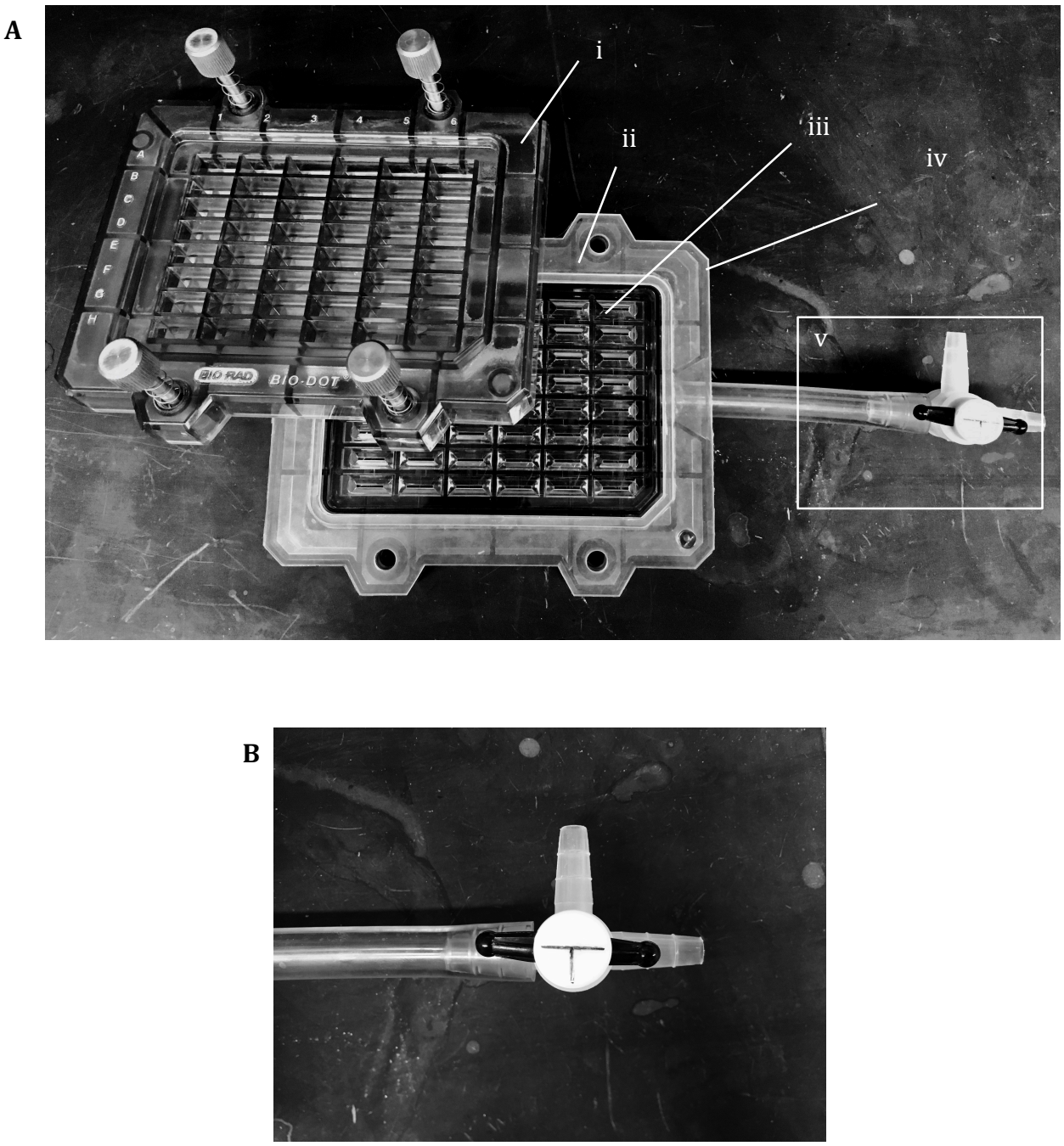

Figure 2. BioRad Bio-Dot SF Microfiltration Apparatus set-up. (A) (i) support template with sealing screws; (ii) sealing gasket; (iii) gasket support plate; (iv) vacuum manifold; (v) tubing and flow valve (BioRad). (B) Flow valve setting 1.

manufacturer guidelines and unused wells were filled with 200 μL $NaHCO_3$ to prevent diffusion across wells. Probes were allowed to incubate for 1 minute at room temperature before the fluid in each well was vacuumed through the blotting membrane. Guide slots were marked on the membrane with ballpoint pen, and the membrane was transferred to a 14 x 14 cm plastic Tupperware container for incubation at room temperature, rocking in 50 mL 100 mM NaOH for 7 minutes. Next, the NaOH was replaced with 50 mL 2x SSPE/0.1% SDS and the membrane was incubated, shaking, for 5 min at 60^{o}C. At this point, if the membrane was not being hybridized the same day, it could be washed with 50 mL 20 mM EDTA (pH 8), rocking at room temperature for 20 minutes. The membrane could then be stored at 4^{o}C in a sealed plastic bag with 10 mL 20 mM EDTA.

For membrane hybridization, 0.5μL biotinylated product was mixed to a total volume of 200 μL with 2x SSPE/0.1% SDS and denatured at 100^{o}C for 10 minutes. The denatured DNA was then immediately placed on ice for 5 minutes. Meanwhile, in a 9 x 13 cm plastic dish, the activated membrane and three pieces of filter paper were soaked in 50 ml 2x SSPE/0.1% SDS for 5 minutes at room temperature. The filter paper and membranes were arranged in the BioRad apparatus as before and excess liquid was vacuumed through with maximum vacuum pressure. 200 μL of denatured sample was loaded into each well and allowed to hybridize for 60 minutes at 42^{o}C. Again, empty wells received 200 μL 2x SSPE/0.1% SDS to prevent cross-flow. After hybridization, any remaining liquid was pulled through the membrane by vacuum, and the membrane was transferred back to the Tupperware container for two washes, both shaking in 50 mL 2x SSPE/0.5% SDS at 52^{o}C for 10 minutes. Next, the membrane was incubated with 10 mL streptavidin-POD-conjugate (diluted 1:4000 in 2x SSPE/0.5% SDS) in a sealed plastic bag at 42^{o}C for 30 minutes. The membrane was then transferred back to the Tupperware container and

washed twice, shaking, in 50 mL 2x SSPE/0.5% at 42°C for 10 minutes each time. The solution was replaced with 50 mL 2x SSPE and the membrane was rocked for another 5 minutes at room temperature. For signal visualization, 1-step tetramethylbenzidine (TMB) substrate for membranes (SeraCare) was pipetted directly onto the membrane until a convex meniscus formed over the surface. Color was allowed to develop for 5 to 10 minutes before rinsing with DI water to stop the reaction. To strip the hybridized samples from the probes, the membrane was washed twice, shaking in 50 mL 1% SDS at 80°C for 30 minutes each. Finally, the membrane was rocked at room temperature for 15 minutes in 20 mM EDTA (pH 8) before being stored at +4°C in a plastic bag with about 10 mL 20 mM EDTA.

IV. Results

1. Sample Characterization

Adult nematode identities were determined by RFLP analysis of NC1-NC2 PCR amplicons using *Dra1* and *Hinf1* Type II restriction endonucleases and comparison to known fragment patterns [29]. *Dra1* only cleaved putative *Trichostrongylus*-derived DNA, and produced a restriction pattern comparable to those of *T. colubriformis* and *T. axei*. *Hinf1* cleaved putative *Teladorsagia* derived DNA, but did not cleave *Trichostrongylus* or *Haemonchus*, confirming the *Teladorsagia* species to be *T. circumcincta* and the *Trichostrongylus* species to be *T. axei.* As expected, neither restriction enzyme was able to cleave *Haemonchus* spp. samples. DNA sequencing confirmed the identities of the adult worms to be *T. axei*, *T. circumcincta*, and *H. contortus,* respectively. To assess the degree of shared sequence identity between the samples and their respective probes, consensus sequences from four adult worms of each genus were compared to the genus-specific probe sequences. nITS2T had 100% shared identity with the *T. axei* consensus, 81% with *T. circumcincta*, and 75% with *H. contortus*; nITS2Te showed 60%

shared identity with *T. axei,* 85% with *T. circumcincta* and 70% with *H. contortus*; and nITS2HC showed 65% shared identity with *T. axei,* 43% with *T. circumcincta*, and 90% with *H. contortus*. With each genus showing the highest degree of shared sequence identity with its respective probe, these findings confirm genus-specificity at the molecular level.

Mixed-infection fecal samples were characterized by McMaster flotation and PNA staining. 16 samples were found to clear the 50 epg threshold and ranged in percent *H. contortus* composition from 4% to 71%. Of these 16 samples, five were selected for additional experimentation, possessing 4%, 24%, 40%, 52%, and 71% *Haemonchus*, respectively. Representative samples were also microscopically found to contain *Eimeria* spp., *Trichuris ovis, Strongyloides papillosus*, *Nematodirus* spp., and *Muellerius capillaris*, confirming the presence of other nematodes that may be amplified by the NC1-NC2 primer set.

To determine if the concentration of NC1-NC2 PCR product varies across genera, four adult worms from each genus were PCR amplified three times. The product concentrations of each run were then averaged by genus. No difference in mean product concentration was observed across genera (ANOVA, $p > 0.05$), allowing for standardization of sample volumes in all genus-specific assays. NC1-NC2 amplified products from 100 egg samples had a significantly lower mean concentration, however, as compared to single adult samples (two-sample t-test, $p < 0.05$).

2. Proof of Concept:

Proof of concept was obtained using the S5-AS5 primer/probe combination. Single adult *H. contortus* amplicons generated a bright blue band when hybridized with the S5 probe, with neither the probe nor the DNA sample producing signals independently. No improvement in hybridization was seen when increasing the PCR product volume from 0.5 μL/well to 5 μL/well

or in varying the probe concentration from 10 pmol/well to 1000 pmol/well (results not shown). DNA concentrations S5-AS5 amplicons were not measured. To assess the effects of amplicon length on hybridization efficiency, biotinylated PCR products were cleaved with *HpyCH4II* for two hours at 37^{o}C. Both restricted and non-restricted DNA fragments were capable of hybridization, and no significant difference in signal was observed (results not shown). In future assays, PCR products were not cleaved prior to hybridization, and the S5 probe was used as a positive control at the original sample volume of 0.5 μL/well with 10 pmol/well probe. The stripping procedure was validated by carrying out a mock hybridization without the addition of sample DNA. No precipitate was formed in any well, and stripped membranes were henceforward used up to ten times before disposal.

2. Genus-Specific Reverse Line-Blot

nITS2HC and nITS2T were both able to detect DNA derived from individual adult worms of their respective genera down to a concentration of 1.44 pmol/well sample. When sample volumes were increased from 0.005 μL/well (0.014 pmol) to 5μL/well (14.4 pmol), there was a marked increase in signal intensity. The strongest signal was produced with 5μL/well, and no signal was observed at 0.005 μL/well. Modest improvements in signal intensity were also observed when increasing probe concentrations from 10 pmol/well to 1000 pmol/well (Figure 3A, 3B). nITS2Te did not produce a signal at the standard protocol concentrations of 0.5μL/well sample and 10 pmol/well probe, and increasing the sample DNA concentration to 5μL/well did not improve the signal. Given the probe concentration's limited effect in the other two genus-specific hybridizations, variations in probe concentration were not attempted.

Given that the maximum signal intensity of nITS2HC and nITS2T was achieved with 5 μL of DNA, and given the lack of signal improvement with increased probe concentrations, the

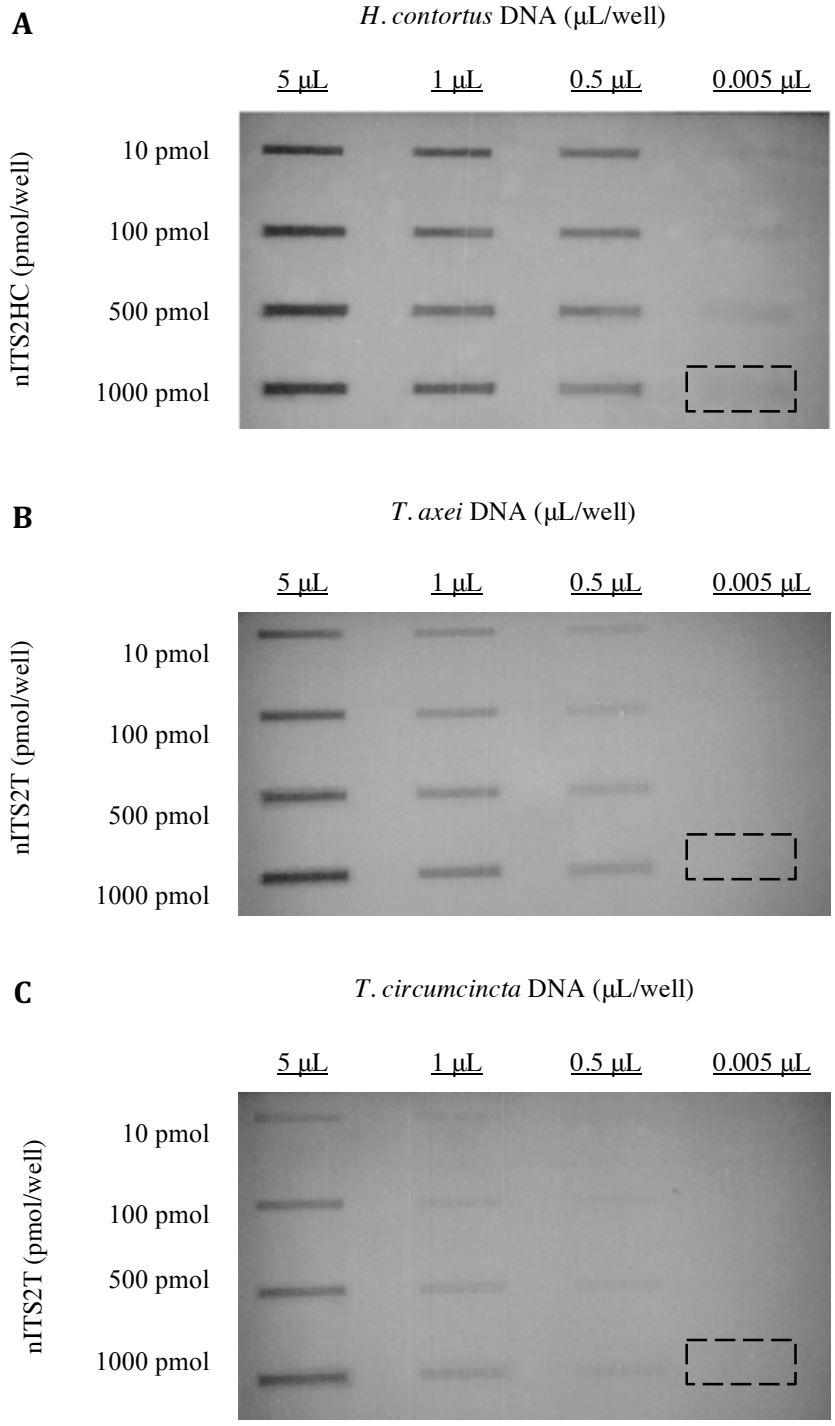

Figure 3. Genus-specific reverse line-blots. Each membrane shows the hybridization of the probe specified to DNA derived from one adult worm of the genus indicated, amplified with the bio-NC1-NC2 primer set. 1 µL of each amplicon in the absence of its respective probe produced no signal (results not shown). Boxed wells indicate negative controls and do not contain sample DNA.

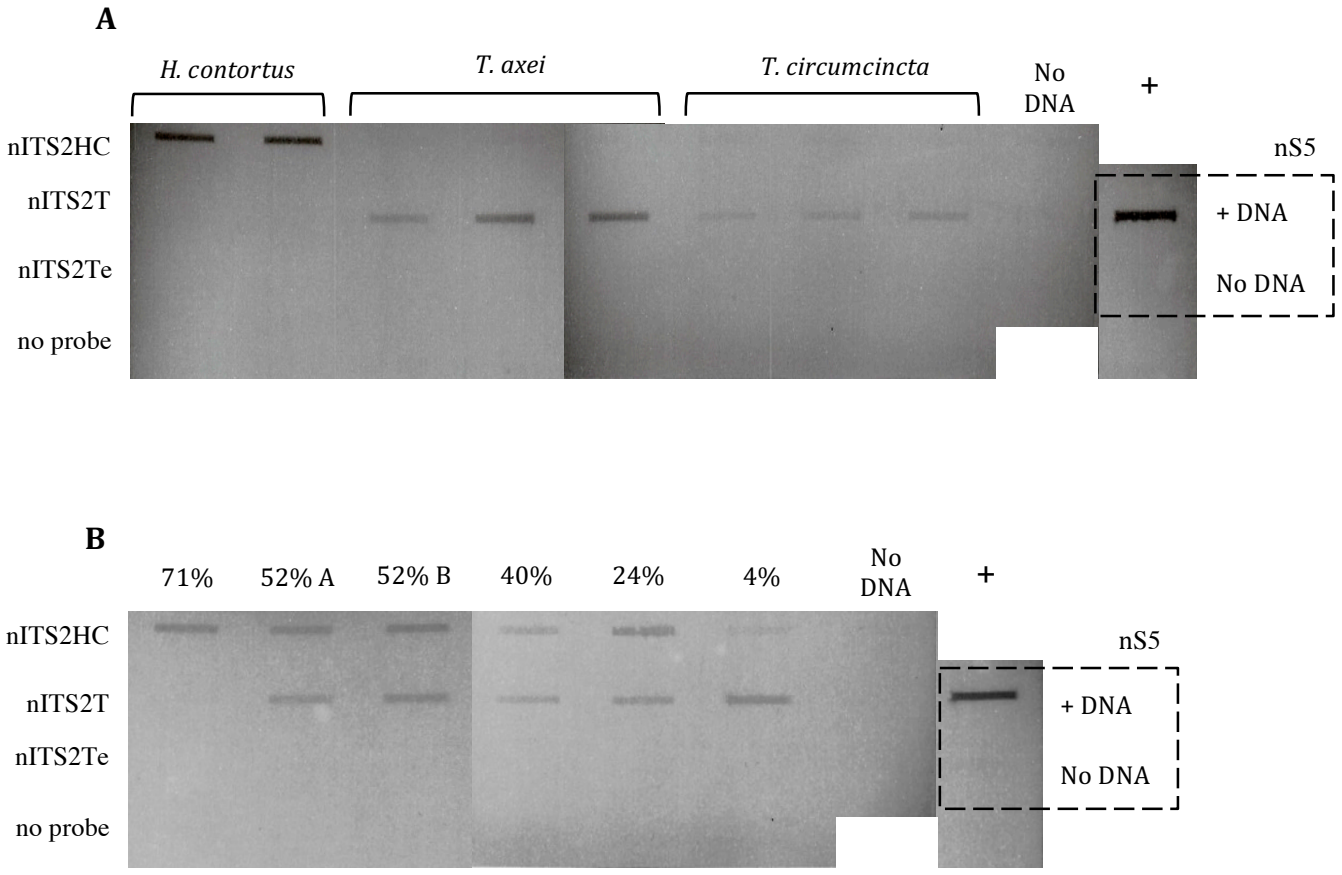

Figure 4. Cross-hybridization reverse line-blots. Spliced for ease of interpretation. Each row contains 10 pmol of the probe indicated. Boxed wells were probed with 10 pmol nS5. The positive control column received 5 µL/well *H. contortus* DNA amplified with the bio-S5-AS5 primer set. (A) Each column received 5 µL/well of DNA derived from a single worm of the species indicated, amplified with the bio-NC1-NC2 primer set. (B) Each column received 5 µL/well of DNA derived from a mixed-infection fecal sample with the percent *H. contortus* content indicated, amplified with the bio-NC1-NC2 primer set. All fecal samples were derived from 100 eggs, with the exception of 52% B, which was derived from 1000 eggs.

cross-hybridization potential of nITS2HC, nITS2T, and nITS2Te was assessed by adding 5μL/well sample to 10 pmol/well of each probe. nITS2HC was shown to be highly specific, producing bright bands only in the presence of *H. contortus* DNA (Figure 4A). Faint bands were produced in all nITS2HC-probed wells, but the signal was no darker than the negative control and deemed to be insignificant. nITS2T yielded visible signals in the presence of both *T. axei* and *T. circumcincta*, and nITS2Te failed to hybridize with DNA from any of the genera. Additional blots were performed to further characterize the degree of cross-hybridization between nITS2T and *T. circumcincta*. To see if the sample volume and probe concentration could be manipulated to drop out the *T. circumcincta* signal while retaining the *T. axei* signal, the nITS2T probe concentration was varied from 10 pmol/well to 100 pmol/well, and the amount of *T. circumcincta* DNA from 5 μL/well to 0.005 μL/well. With the exception of a loss of signal when 0.5μL DNA was hybridized to 10 pmol probe, the pattern of hybridization closely resembled that of *T. axei* (Figure 3C). To assess the effects of hybridization temperature on probe specificity, another line blot was carried out at 60^{o}C. 5μL/well PCR product of either *T. circumcincta* or *T. axei* was exposed to varying concentrations of nITS2T, as before. All wells saw an increase in signal intensity relative to hybridizations of the same membrane at 42^{o}C. Although *T. circumcincta* consistently produced fainter signals when compared to *T. axei*, both species exhibited the same basic hybridization pattern regardless of hybridization temperature (Figure 5).

3. Relative Sensitivity of nITS2HC

To assess the probes' ability to discriminate between eggs in a mixed-infection fecal sample, 5μL/well (7.98 pmol) of PCR product from five fecal samples ranging in their percent *H. contortus* content from 4% to 71% were added to 10 pmol/well of each genus-specific probe.

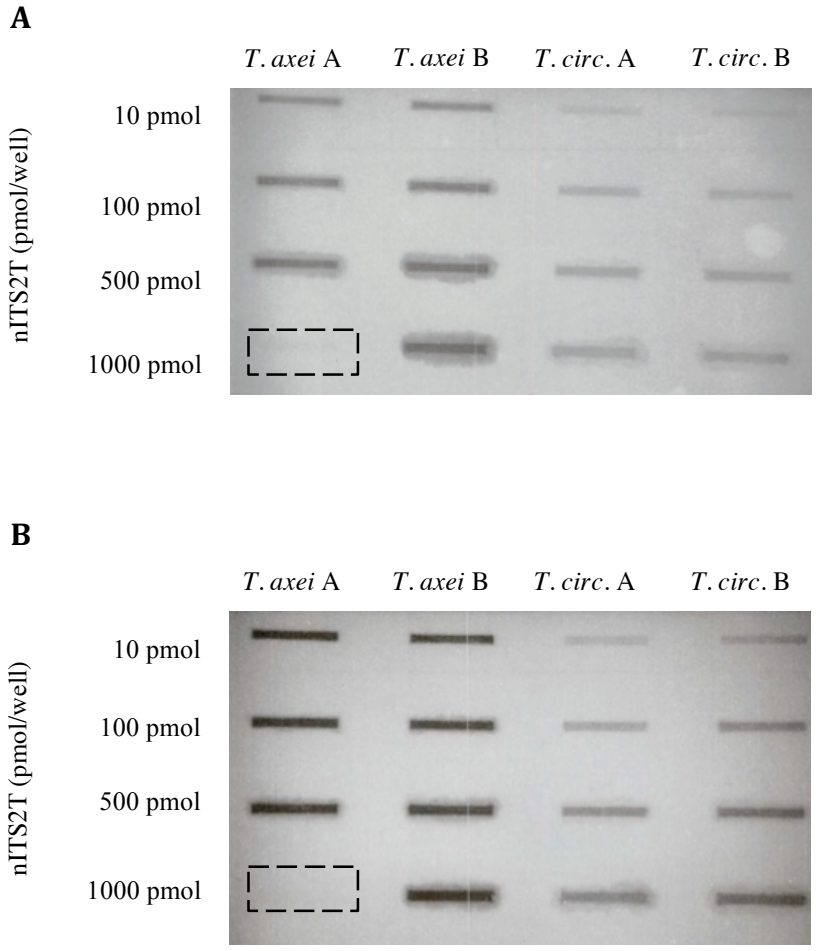

Figure 5. nITS2T reverse line-blot comparing *T. axei* and *T. circumcincta* signal intensity at a hybridization temperature of (A) 42°C or (B) 60°C. Each column contains 5 μL/well DNA derived from an adult worm of the genus indicated, amplified with the bio-NC1-NC2 primer set. Pooled samples consisting of 2.5 μL of tagged product derived from each worm generated no signal in the absence of a nITS2T (results not shown). Boxed wells indicate negative controls and do not contain sample DNA.

Sample volumes and probe concentrations were determined using the genus-specific probe optimization described above. The use of a larger sample volume to account for differences in product concentration was not attempted due to volumetric constraints of the amplification reaction. The sample with the highest proportion of *H. contortus* produced a faint signal when hybridized with nITS2HC, and no signal when hybridized with nITS2T. Conversely, the sample with the lowest proportion of *H. contortus* produced a faint signal when hybridized with nITS2T and little to no signal when hybridized with nITS2HC. Intermediate proportions of *H. contortus* hybridized to both nITS2HC and nITS2T, however differences in signal intensity were difficult to determine. Increasing the number of eggs from 100 to 1000 did not significantly enhance signal intensity (Figure 4B).

V. Discussion

In this study, I have attempted to detect DNA from three different genera of nematodes using reverse line-blot hybridization and probes derived from genus-specific primers. Reverse line-blot hybridization has been praised in the past for its incredible versatility, low cost, high throughput, and reasonable turnaround times, and has been shown to exhibit specificity down to the species level using probes complementary to the IGS and ITS regions [24,35,37]. Although the study here uses a single primer set for amplification in multiple organisms, assay versatility can be enhanced further through the use of multiplex PCR (mPCR). In such an assay, probes would not be restricted to a single gene or transcribed spacer, but instead could target multiple attributes throughout the genomes of one or more organisms [24]. This tremendous versatility has clear implications for diagnostics, and the technique has been explored for this purpose in a number of other studies. In 2007, Zeng et al. developed species-specific probes, also targeting the ITS-1 and ITS-2 regions, for the diagnosis of invasive fungal infections. Probes

complementary to both regions were shown to be highly sensitive, and capable of correct diagnosis 85.7% of the time when compared to fungal cultures. Similarly, in a study by Wang et al. (2014), both RT-PCR and mPCR/RLB were found to be significantly more sensitive in the identification of pathogens as compared to standard bacterial culturing methods. Furthermore, although RT-PCR was capable of detecting as little as 2 fg of genomic DNA, mPCR/RLB had better agreement with bacterial culture results and was better suited for higher throughput analyses [38]. These findings are highly encouraging and maintain reverse line blot hybridization as a promising avenue for future diagnostic assays.

Most notably, this study showed that nITS2HC is capable of sensitive and specific detection of *H. contortus* DNA. Additional research is required, however, before these results can be translated into quantitative estimates of worm burden. Still, given *H. contortus*'s remarkable fecundity and ability to provoke severe anemia in animals harboring fewer than 100 worms, even a qualitative assay would provide vital information for the management of *H. contortus* infections [39]. While the other two probes lacked the same degree of specificity, nITS2T may still have diagnostic relevance. Although other parasites can be found in the small and large intestines that may complicate the problem of cross-hybridization, *H. contortus, Trichostrongylus* spp., and *T. circumcincta* are the three most common gastrointestinal parasites of sheep [9]. Moreover, *Trichostrongylus* and *Haemonchus* are the two genera of greatest pathogenic importance [5,12]. As such, even a cross-hybridizing probe may provide insight into the extent and severity of infection. nITS2HC and nITS2T probes may therefore be portrayed as "*Haemonchus*" and "non-*Haemonchus*" probes, respectively, paralleling the function of a McMasters flotation and PNA stain, described above. In this classic assay pairing, the total strongyle epg is calculated before fluorescent staining determines what percentage of that total is

Haemonchus. Similarly, if I can quantify the results of RLB by calculating the amount of precipitate formed at various DNA concentrations, the amount of *Haemonchus* can either be considered relative to other major abomasal parasites, or directly inferred from the intensity of the bands. Such improvements are tremendously attractive from a diagnostic perspective. Where lectin staining requires hours of microscopic observation beyond the already lengthy staining procedure, RLBH would allow for the simultaneous processing of up to 45 samples, dramatically reducing the amount of time needed to reach a diagnosis.

Although nITS2T may have utility as a cross-hybridizing probe, the question remains why it lacked the genus-level resolution it possessed in PCR. Clearly, differences in experimental conditions between blot hybridization and PCR amplification have a dramatic effect on hybrid stability. Still, Kong & Gilbert (2006) recommend similar parameters for both primer and probe design. That is to say, primers and probes should have a length of 18-30 bp and a melting temperature (T_m) of 58-65^{o}C, approaching both their PCR annealing temperature and hybridization temperature (T_h) [33]. The specificity of the probes may also be impacted by both the T_h and the stringency of post-hybridization wash conditions [33,40]. Perhaps un-intuitively, increasing the hybridization temperature from 42^{o}C to 60^{o}C did not eliminate the non-specific binding of nITS2T, but rather improved hybridization efficiency. As such, additional investigation into the effects of wash stringency may be a valuable next step. The cross-hybridization of nITS2T is less surprising when we consider the degree of similarity with both *T. axei* and *T. circumcincta*. Although entirely identical to the *T. axei* consensus sequence, nITS2T shared a surprising 13 consecutive bases and 81% shared identity overall with the *T. circumcincta* consensus sequence. In fact, it is recommended that oligonucleotide probes have no more than 70% shared identity or 8 consecutive shared bases with non-target regions in order to

maintain target-specificity [40]. Although our consensus sequences were constructed with a very limited sample number, I hypothesize that this high degree of shared sequence identity may account for the cross-hybridization reported here. The incomplete shared identity between *T. circumcincta* and nITS2T may have also contributed to its decrease in signal intensity relative to hybridizations between nITS2T and *T. axei.* To improve specificity, the probe itself could be modified slightly. Although a probe's central region is one of the primary determinants of probe specificity, the bases at the 3' end can also play a role in differentiating between samples [33]. Extending the 3' end of the nITS2T probe by two bases would incorporate an additional two mismatches, and may aid in genus-specific resolution. Still, this may not be enough to overcome the high degree of similarity in the central region.

What sequence identity cannot explain, however, is nITS2Te's inability to detect worms of any genus, despite sharing 85% of its sequence with the *T. circumcincta* consensus. Lengthening the probe may increase sensitivity [41], albeit at the risk of decreasing the probe's specificity, but restrictions on optimal RLB probe lengths may limit the impact of such a change [33]. Seeing as this probe was already designed to maximize genus specificity within the ITS-2 region, improvements to sensitivity would therefore require either the use of another gene target or modifications to the hybridization conditions. Given nITS2Te's low T_m as compared to each of the other probes, it may be valuable to again consider the stringency of the wash steps post-hybridization. In the most stringent wash, the membrane is shaken in 2x SSPE/0.5% SDS for 10 minutes at 52^{o}C. This temperature closely approximates the predicted T_m of the nITS2Te probe. Given that the probe incompletely matches the *T. circumcincta* consensus, this temperature may have proven too stringent for hybridization. Future experiments may consider lowering the post-

hybridization wash temperature and/or decreasing the percentage of SDS in the washing solution to assess the effects of wash stringency on probe hybridization.

In addition to hybrid stability, differences in PCR product concentration and gene copy number may also have an effect on signal intensity. Within a genus, I have demonstrated that signal intensity is directly correlated with sample concentration, with each genus-specific probe showing a decrease in signal intensity with decreasing DNA concentration. Unfortunately, absolute measures of signal intensity were not made and so additional work will need to be done to more precisely quantify this relationship. Samples that produced noticeably fainter bands in gel electrophoresis also showed reduced signal intensity as compared to other members of the same genus. Although the absolute concentration of PCR product was not shown to vary significantly between species, ITS-2 copy number has been shown previously to vary between Trichostrongylids. In a study by von Samson-Himmelstjerna et al. (2002), first-stage larvae of *H. contortus*, *T. colubriformis,* and *Ostertagia leptospicularis* were shown to possess 1.37×10^6, 2.88×10^5, and 2.56×10^5 copies of ITS-2, respectively. Assuming increased copy number provides additional targets for hybridization, a similar pattern may be expected in the signal intensities of each genus-specific hybridization. In fact, this is exactly what was observed, with *T. axei* and *T. circumcincta* producing noticeably fainter signals when hybridized with nITS2T relative to the same concentration of *H. contortus* hybridized with nITS2HC. It is unclear why this difference in copy number was not reflected in my calculations of PCR product concentration, but it is likely due to the small sample size and slight experimental variations between amplifications.

Most of the optimization presented here depends on the use of adult worms; however, because adults are only available through necropsy, preventative diagnostics will require the use

of other life stages. Given the limitations of larval culturing techniques [21], eggs are the most likely targets for future hybridization analyses. Although their accessibility in the feces makes them ideal for diagnostic work, the use of eggs presents two major problems for assay optimization: (*i*) differences in PCR amplification depend on the degree of egg development and (*ii*) the exact species composition of the samples is unknown. Variations in PCR amplification has been reported by others [26,43], with Schnieder et al. (1999) reporting significant increases in PCR product yields after as little as 24 hours of development. The use of more mature organisms will therefore result in higher PCR yields and enhanced qualitative detection of strongyles present at lower frequencies. Unfortunately, in allowing eggs to develop, one also increases the developmental variation within the sample, potentially introducing errors in quantitative and semiquantitative evaluations of DNA concentration [43]. In an effort to limit this variability, the present study stored all fecal samples at +6^{o}C for 4 days before DNA extraction. Although storage at this temperature has been shown to keep equine strongyle eggs from hatching [44], embryonation is possible down to +4^{o}C [44,45]. As such, closer attention to sample storage conditions and processing times may greatly impact our ability to create a reproducible, quantitative assay.

The second obstacle to the use of mixed egg samples stems from the diversity of organisms present in a given sample. The protocol described here uses a single primer set for the amplification of multiple homologous templates. Although this experimental design is desirable for its simplicity and ease of interpretation, the technique is susceptible to considerable competition between templates. Slight differences in template frequency, primary DNA structure, and reaction conditions can dramatically impact the probability of a given template's amplification, greatly reducing PCR sensitivity [46]. As such, the semi-quantitative assay

presented here may have been skewed towards more prevalent templates, obscuring trends in samples with intermediate proportions of *H. contortus.* This multi-template design will also require further investigation into the cross-hybridization of probes with other genera amplified by the NC1-NC2 primer set. Although lectin staining asserts with relative certainty that a portion of the observed signal was due to *H. contortus* DNA, it is unclear whether the presence of additional parasites contributed to that signal. Similarly, with no way to determine if *Trichostrongylus* or *Teladorsagia* were present in these fecal samples, no conclusions can be drawn regarding nITS2T's sensitivity to fecal samples of varying parasite composition. Rather, all that can be said with certainty is that strongyles other than *Haemonchus* were present in the sample. Future experiments should therefore assess both probe sensitivity and specificity, testing hybridization with known mixtures of DNA extracted from *Haemonchus, Trichostrongylus,* and *Teladorsagia*.

The choice of signal visualization method can also greatly impact assay sensitivity. DNA blotting techniques, including reverse line-blots, Southern blots, and slot/dot blots, were originally visualized using radiolabeled probes, but in recent years there has been increased interest in colorimetric and chemiluminescent detection methods. These techniques provide safer alternatives to radioactive isotopes and have the advantage of greater stability and longer shelf-lives [25]. Both colorimetric and chemiluminescent detection methods require the sample to first be labeled with either biotin or digoxigenin (DIG). Colorimetric visualization is then achieved by indirect immunofluorescence or conjugation with a fluorochrome or enzyme to produce a colored precipitate [25]. Alternatively, chemiluminescent detection relies on light emitted by the cleavage of a chemiluminescent dioxetane substrate by a conjugated alkaline phosphatase, and is generally preferred for its increased sensitivity over colorimetric detection methods [47]. Still,

reasonably high degrees of sensitivity have been achieved using colorimetric detection methods. In fact, Wang et al. (2006) reported 94.3% sensitivity using DIG-labeled probes, anti-digoxigenin alkaline phosphatase conjugated antibodies, and nitroblue-tetrazolium and 5-bromo-4-chloro3-indolyl-phosphate (NBT/BCIP) substrate. Colorimetric detection also has the added benefit of requiring little to no additional instrumentation, making the technique considerably more accessible and attractive for diagnostic applications. As such, although additional investigations into assay sensitivity are required, colorimetric detection remains a viable mechanism for signal visualization.

Once this assay has been fully optimized, it may be desirable to explore the use of other blotting platforms. Although the BioRad Bio-Dot SF Microfiltration Apparatus was well-suited for the scale of the present study, other apparatuses exist that may be more appropriate for higher-throughput analyses. Of particular interest is the Immunetics Miniblotter [33-35]. Capable of the simultaneous hybridization of up to 45 samples, this apparatus would afford tremendous experimental versatility over the current platform. Still, there are some key differences between the Bio-Dot SF and the Miniblotter that will need to be considered in making this transition. Most striking is the Bio-Dot's use of vacuum filtration. While the Immunetics Miniblotter removes excess liquid by aspiration from the surface of the membrane, the Bio-Dot SF pulls excess sample through the membrane into the vacuum manifold (Figure 2). Although the effect of vacuum aspiration on hybridization efficiency will need to be investigated further, the use of vacuum filtration for the transfer of DNA onto a membrane has been studied in the past. In a study by Gross et al. (1988), it was shown that vacuum filtration did not impact the efficiency of DNA transfer from a polyacrylamide gel to a nylon membrane. Furthermore, the specific vacuum strength was not critical for successful transfer [49]. As such, the use of vacuum

filtration in the present study is unlikely to affect the translation of these findings to other blotting apparatuses. The second most striking difference between these platforms is the way in which samples and probes are applied to the membrane. Unlike the Bio-Dot apparatus, which requires the individual inoculation of every well, each of the Miniblotter's channels allows for the simultaneous preparation of 45 different hybridization reactions. This setup will dramatically reduce assay handling time, although additional optimization will be required to account for this difference in sample distribution.

Reverse line-blot hybridization has a promising future in the management of small ruminants, both in the genus-specific detection explored here and in the detection of specific SNPs associated with resistance. Patterns of benzimidazole resistance are particularly well-characterized and would lend themselves well to such an assay. At this point in time, three resistance SNPs have been identified in the β-tubulin isotype 1 gene, all of which have been detected in one or more of the genera described here. The SNP at codon 200 results from the substitution of a phenylalanine with tyrosine (F200Y) and is the dominant BZ-resistance marker in *H. contortus, T. colubriformis,* and *T. circumcincta* [50]. It has also been found, to a lesser extent, in populations of *T. axei* [51]. A second SNP (F167Y) has been found in *H. contortus* and *T. circumcincta*, and a potential third (E198A) has now been detected in *H. contortus* [50]. Other studies have had success in the past with the detection of single-nucleotide polymorphisms with RLBH. Bunschoten et al. (2000) developed an assay for the simultaneous detection of polymorphisms in the human *N*-acetyltransferase genes, *NAT1* and *NAT2,* despite high degrees of similarities between the two. They further determined that the RLBH technique was in complete agreement with allele-specific PCR and PCR-RFLP methods. Echoing these results, a study by Shah et al. (2016) successfully used mPCR/RLBH for the genotyping of 17 biallelic

sites in 11 different genes associated with coronary artery disease, again with complete agreement with sequencing results. Both of these studies highlight RLBH's remarkable specificity and inspire hope that it could be used to shed even more light on the extent of anthelmintic resistance in small ruminant populations.

VI. Conclusion

This study saw mixed success in the detection of three nematode genera of veterinary importance. Of the genus-specific primers published by Schnieder et al. (1999), only the *H. contortus*-specific forward primer was suitable for genus-specific hybridization applications. The *T. axei* forward primer, although cross-hybridizing with both *Trichostrongylus* and *Teladorsagia*, remains a useful diagnostic tool. Together, the *Haemonchus* and *Trichostrongylus* probes allow for the detection of all three of the major abomasal parasites of small ruminants. To improve the assay's utility, additional research is needed to better characterize the effects of hybridization temperature and wash stringency on probe specificity. Additional optimization is also required for use of this assay with helminth eggs and larvae, and for translation of the technique to other blotting apparatuses. The reverse line-blot technique has had great success both in other species-specific and SNP-specific applications, and with the ever-growing threat of anthelmintic resistance, veterinarians would benefit greatly from the ability to detect resistant organisms prior to treatment. It is therefore my hope that this technique will provide the versatility, high throughput, and specificity so desperately needed to revolutionize the treatment of small ruminants.

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